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Genetic Exchange by Recombination or Reassortment Is Infrequent in Natural Populations of a Tripartite RNA Plant Virus

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Two hundred seventeen field isolates of cucumber mosaic cucumovirus (CMV), sampled from 11 natural populations, were typed by RNase protection assay (RPA) using probes from the genomic RNAs of strains in subgroup I and in subgroup II of CMV strains. Most (85%) of the analyzed isolates belonged to subgroup I. For these subgroup I isolates, only two clearly different RPA patterns, A and B, were found for each of four probes representing RNA1, RNA2, and each of the two open reading frames in RNA3. On the basis of these RPA patterns for each probe, different haplotypes were defined. The frequency composition for these haplotypes differed for the various analyzed populations, with no correlation with place or year of sampling. This genetic structure corresponds to a metapopulation with local extinctions and recolonizations. Most subgroup I isolates (73%) belonged to haplotypes with RPA pattern A (type 1) or B (type 2) for all four probes. A significant fraction of subgroup I isolates (16%) gave evidence of mixed infections with these two main types, from which genetic exchange could occur. Genetic exchange by segment reassortment was seen to occur: the fraction of reassortant isolates was 4%, reassortment did not occur at random, and reassortants did not become established in the population. Thus, there is evidence of selection against reassortment between types 1 and 2 of subgroup I isolates. Aphid transmission experiments with plants doubly infected with type 1 and type 2 isolates gave further evidence that reassortment is selected against in CMV. Genetic exchange by recombination was detected for RNA3, for which two RPA probes were used. Recombinant isolates amounted to 7% and also did not become established in CMV populations. Sequence analyses of regions of RNA1, RNA2, and RNA3 showed that there are strong constraints to maintain the encoded sequence and also gave evidence that these constraints may have been different during divergence of types 1 and 2 and, later on, during diversification of these two types. Constraints to the evolution of encoded proteins may be related to selection against genetic exchange. Our data, thus, do not favor current hypotheses that explain the evolution of multipartite viral genomes to promote genetic exchange.

Multipartite (i.e., multicomponent) RNA viruses, i.e., viruses that encapsidate separately the segments of a divided genome, pose interesting questions on virus evolution. For a multipartite virus to initiate a successful infection, a set of viral particles containing the complete genome must infect the same host cell. This represents an obvious biological cost that must be compensated for somehow for multipartitism to have evolved. During the last few years, two main hypotheses regarding the advantages of a multipartite genome have been proposed. One hypothesis proposes that multipartitism has evolved to favor genetic exchange by reassortment (i.e., pseudorecombination) (6, 8, 45). Genetic exchange would counterweight the fitness losses due to the accumulation of deleterious mutations through the effect known as Müller's ratchet (32). This effect is thought to be particularly high for RNA genomes (6, 9) due to high error rates of RNA replication (14). Of course, this hypothesis must assume that genetic exchange through recombination does not occur or is very infrequent for RNA genomes. A second hypothesis states that the faster replication rates of smaller RNA segments, compared with the hypothetical undivided genome, would result in intracellular selection for smaller RNAs and lead to the evolution of multipartitism (34, 35). Passage experiments have indeed shown that Müller's ratchet operates on RNA viruses (7, 15, 37) and that reassortment of genomic segments may reverse its effects (10). Also, reassortant viruses have been found in natural populations of animal viruses with segmented genomes (20, 33) and of plant viruses with multipartite genomes (48, 56). Nevertheless, the first of these two hypotheses would be best tested through analyses of natural populations of multipartite viruses that would allow an estimation of the frequencies of genetic exchange by reassortment and by recombination. To our knowledge, these studies are lacking.

We present here such an analysis of natural populations of the tripartite plant virus cucumber mosaic cucumovirus (CMV). CMV is a particularly successful virus: it is found all over the world, it has a very large host range, it is found at high incidences in many of its natural hosts, and it is efficiently transmitted in a nonpersistent manner by many species of aphids. CMV is endemic in Spain, causing epidemic outbreaks in most horticultural crops from spring to fall (29). CMV has been extensively studied (see reference 40 for a review). The two larger genomic segments, RNA1 and RNA2, encode proteins (p1a and p2a) that are part of the viral RNA replicase complex (19). RNA3 encodes the movement protein (MP) (24) and the coat protein (CP). MP, CP, and a second protein encoded in RNA2 (p2b) are required for virus colonization of the host plant. CP, in addition to this function and its structural role, has determinants for symptom induction and for aphid

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transmission of the virus (44, 51). RNA1 and RNA2 are each encapsidated in separate particles, while RNA3 is coencapsidated with RNA4, the subgenomic mRNA for CP, in a third particle.

The data reported here allow the quantification of the frequency of mixed infections by different strains of CMV and of reassortant and recombinant isolates. It is shown that reassortant genomes are not more frequent that recombinants. In addition to their relevance in understanding virus evolution, these data are relevant for risk assessment of the use of transgenic plants with viral genes for resistance to viruses, another aspect of consequence in virology.

MATERIALS AND METHODS

Virus isolates. A total of 217 CMV isolates representing 11 field populations, were analyzed. We call a population a set of isolates present during one growing season in the same area. Populations were sampled at areas near Valencia (VAL; eastern Spain) in June 1989, 1990, 1991, 1994, and 1995; Barcelona (BAR; northeastern Spain) in September 1992 or during the summers (June to September) of 1993 and 1994; Leon (LEO; northwestern Spain) in October 1993; and Madrid (MAD; central Spain) during the summers of 1993 and 1994. The minimum distance between these areas was 350 km. The distance between sampled fields from an area was less than 20 km. Sampled hosts included tomato (for the VAL, BAR, and MAD93 populations), pepper (for the LEO93 and MAD94 populations), melon, zucchini, or cucumber (for MAD93 and MAD94), bean (for MAD94), and local weeds (*Convolvulus arvensis*, *Parietaria officinalis*, and *Portulaca oleracea*) for BAR93. Except for weeds, only symptomatic plants were sampled.

We call an isolate a virus preparation derived from a single field-infected plant. Isolates were named with the locality name, the first letter from the original host plant name (e.g., T = tomato and M = melon), the year of the sample, and an ordinal (e.g., BAR.T93/24 or VAL.T89/17). Note that ordinals need not be consecutive, since not all of the sampled field plants were infected with CMV. Whenever possible, CMV virions were directly purified from field-infected material, but if this material was not enough, sap from the original plant was used to inoculate *Nicotiana tabacum* cv. Xanthi-nc plants, from which virions were purified. Isolates from the oldest populations had to be multiplied in the course of this work, and in no case did a single-passage multiplication in tobacco result in a change of the RNase protection assay (RPA) pattern (see below) from the original preparation made out of field-infected material. Virion purification was always done as described by Lot et al. (28), and virion RNA was extracted with phenol and sodium dodecyl sulfate.

Genetic characterization of viral isolates. CMV isolates were characterized by RPA using probes complementary to regions of the genomic RNAs (gRNAs). RPA was done as described by Aranda et al. (1, 2). Four different probes were derived from cDNA clones complementary to the gRNA of strain Fny-CMV, exemplifying (a gift from P. Palukaitis, Cornell University, Ithaca, N.Y.), subgroup I of CMV strains: probe I.1, complementary to nucleotides 851 to 1694 of RNA1; probe I.2, complementary to nucleotides 1210 to 2139 of RNA2; probe I.3, complementary to nucleotides 250 to 706 of RNA3, in the MP open reading frame (ORF); and probe I.4, complementary to nucleotides 1389 to 1840 of RNA3, in the CP ORF (nucleotides numbered as in references 46, 47, and 39 for RNA2, -1, and -3, respectively). A second set of probes was derived from cDNA clones of the gRNA of strain Ls-CMV, (a gift from P. Palukaitis), exemplifying subgroup II of CMV strains: probe II.1, complementary to nucleotides ~400 to 1400 of RNA1; probe II.2, complementary to nucleotides ~1000 to 2000 of RNA2; and probe II.3, complementary to nucleotides ~1200 to 2000 of RNA3. More precise boundaries for these probes cannot be given, as the nucleotide sequences of Ls-CMV gRNA have not been reported.

Nucleotide sequences were determined by dideoxy nucleotide triphosphate termination of reverse transcription from the gRNAs as described by Fichot and Girard (16). The sequences at two regions were determined: (i) nucleotides 1159 to 1435 in RNA1, using as the primer an oligonucleotide complementary to nucleotides 1469 to 1486 of Fny-CMV RNA1; and (ii) nucleotides 1370 to 1680 in RNA2, using as the primer an oligonucleotide complementary to nucleotides 1712 to 1730 in Fny-CMV RNA2. The sequenced regions were chosen at random.

Genetic diversities were estimated from sequence data as described by Pamilo and Bianchi (41) and by Li (27) (PBL method), using the program Matdisli from Andrey Zharkikh (University of Texas, Houston).

Aphid transmission experiments. A clone of *Myzus persicae* Sulzer, a gift from A. Fereres (Consejo Superior de Investigaciones Científicas, Madrid, Spain), was maintained in healthy pepper plants. For transmission experiments, apterae females of the same age were used. Transmissions were done as described by Perry and Francki (42), using groups of three aphids per plant. Tomato leaves inoculated 5 days before transmission with equal amounts (200 μg of gRNA per ml in 0.1M Na_2HPO_4) of isolates VAL.T89/21 and VAL.T89/15, of haplotypes 1 and 2, respectively (see below), were used as virus source for the aphids. At the time of acquisition, it was checked by RPA that RNA1, -2 and -3 of both haplotypes were found in similar amounts in the source leaves where aphids were allowed to acquire the virus.

RESULTS

Genetic structure of CMV populations. The genetic characterization of 217 CMV isolates representing 11 field populations was done by RPA using three probes derived from Ls-CMV (in subgroup II of CMV strains) and four probes derived from Fny-CMV (in subgroup I of CMV strains). RPA of probes derived from Fny-CMV or from Ls-CMV allow the unequivocal classification of unknown CMV isolates to either subgroup I or subgroup II of CMV strains (reference 38 and Fig. 1): assays of gRNAs with a probe from the heterologous subgroup result in lanes with no protected fragments larger than about 50 nucleotides. On the basis of RPA, it was found that only 32 of the 217 isolates belonged to subgroup II (Table 1). All subgroup II isolates were sampled in the autumn, and most of them came from northwestern Spain (LEO93), although subgroup II isolates were also found in other populations (BAR92, BAR93, BAR94, and MAD93). Only one RPA pattern was found for each of probes II.1, II.2, and II.3 for all subgroup II isolates (not shown); thus, only one genetic type (haplotype) could be defined for these isolates.

Most (85%) of the analyzed CMV isolates belonged to subgroup I of CMV strains. For each of the four Fny-CMV-derived probes, two clearly different RPA patterns were distinguishable: pattern A, in which large fragments similar in size to the probe were protected, and pattern B, in which only smaller fragments were protected (compare, as an example, lanes 12 and lanes 14 in each panel of Fig. 1). These patterns were found in subgroup I isolates of all analyzed populations, irrespective of the species of host plant. No intermediate RPA patterns were found for any of the analyzed isolates. Not only were RPA patterns A and B easily distinguishable, but mixes of both of them, representing double infections of the original field source plant, also were easy to identify (type 3 in Table 1; e.g., lanes 11 in the four panels of Fig. 1).

RPA pattern A or B, or a mixed pattern AB, for each of probes I.1, I.2, I.3, and I.4, was used to define haplotypes within subgroup I isolates. Seventeen different types were so defined. The frequency of each haplotype in each of the 11 populations is shown in Table 1. The genetic structure of these populations was compared based on these frequencies. It was found (Table 1) that although populations differed in their geographical origin and in the year in which they were sampled, no spatial or a temporal pattern of genetic structure could be identified. Also, the haplotype of an isolate was not related to the species of host plant from which it was obtained (not shown).

Genetic exchange through reassortment. Table 1 shows that the more frequent haplotypes of subgroup I isolates were type 1 (27% of isolates) and type 2 (46% of isolates), that is, types in which the RPA pattern is A or B for each of the four assayed probes. Types 3 to 10 represent mixed infections with isolates belonging to type 1 or 2, in which both types coexist for the three CMV gRNAs (type 3) or either pattern A or pattern B has outcompeted the other for RNA1, -2, or -3 (types 4 to 10). We interpret types 4 to 10 as due to mixed infections because on serial passages on tobacco of isolates with these types, an originally undetected A- or B-type gRNA was found or, conversely, an -A- or B-type gRNA could outcompete the other when both were originally found (not shown). Types representing mixed infections were found, on the whole, for 16% of the subgroup I isolates. The displacement of one of the RPA

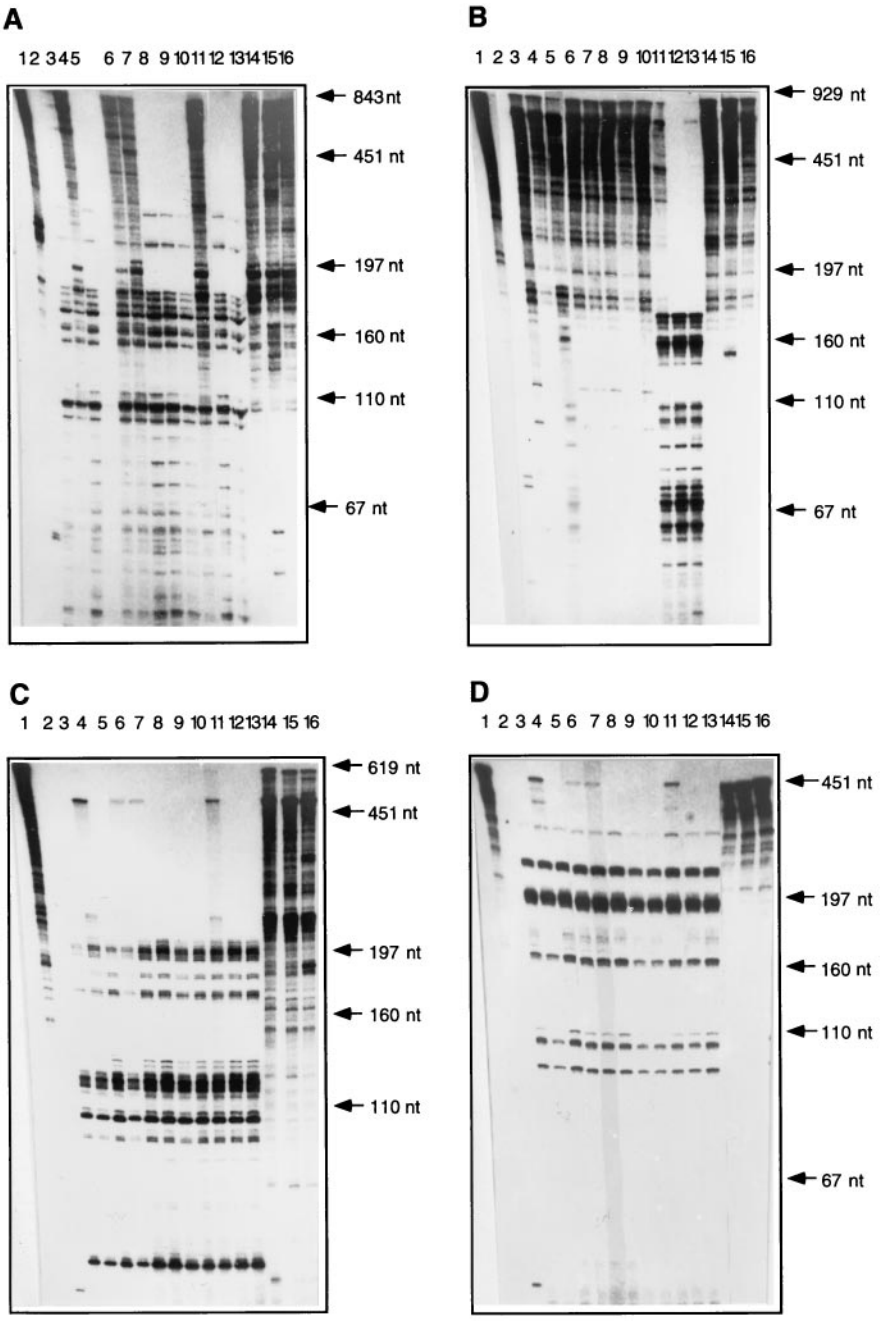

FIG. 1. RPA patterns of CMV field isolates from population VAL89. RPAs were done with probes I.1 (A), I.2 (B), I.3 (C), and I.4 (D). Lanes 1 to 16 present patterns for the following isolates (with the indicated RPA patterns for RNA1, -2, and -3): lane 1, Fny-CMV; lane 2, Ls-CMV; lane 3, T89/1 (B,A,B-B); lane 4, T89/2 (AB,A,AB-AB); lane 5, T89/3 (B,AB,B-B); lane 6, T89/5 (AB,A,AB-AB); lane 7, T89/6 (AB,A,AB-AB); lane 8, T89/7 (B,A,B-B); lane 9, T89/9 (B,A,B-B); lane 10, T89/10 (B,A,B-B); lane 11, T89/13 (AB,AB,AB-AB); lane 12, T89/15 (B,B,B-B); lane 13, T89/16 (B,AB,B-B); lane 14, T89/17 (A,A,A-A); lane 15, T89/19 (A,A,A-A); lane 16, T89/20 (A,A,A-A). The electrophoretic mobility of molecular size markers is indicated to the right of each panel. nt, nucleotides.

pattern types by the other for any of RNA1, -2, and -3 may lead to the appearance of reassortant genomes, represented by types 11 to 14 (see also lanes 8 to 10 in Fig. 1). Reassortant types amounted to 4% of the subgroup I isolates. Each reassortant type, 11, 12, 13, or 14, was found in only one population. Thus, when reassortant genomes between types 1 and 2 appeared, they did not seem to compete efficiently with the parental types. Moreover, the combinations of the two forms (A or B) of each of the three gRNAs did not occur at random, since only six of the eight possible combinations were found (types 1, 2, and 11 to 14), all at very different frequencies.

To determine whether these data could reflect an environment-associated selection against reassortants, the frequency of reassortant appearance under controlled conditions was analyzed. For this, aphid transmissions from tomato leaves supporting an equivalent amount of infection by CMV isolates of types 1 and 2 were done. Transmissions were done with aphid groups of a size (three aphids/plant) such that, based on the

TABLE 1. Genetic structures of 11 natural populations of CMV

Haplotype[a]	RPA pattern			No. of isolates (frequency)											
	RNA1	RNA2	RNA3[b]	VAL89	VAL90	VAL91	VAL94	VAL95	BAR92	BAR93	BAR94	MAD93	MAD94	LEO93	Total
Subgroup II									1 (0.03)	1 (0.03)	5 (0.31)	1 (0.04)		24 (1.00)	32 (0.15)
Subgroup I															
Type 1	A	A	A-A	6 (0.35)		8 (0.73)		4 (0.57)	4 (0.11)	2 (0.05)	1 (0.06)	14 (0.56)	10 (0.63)		49 (0.22)
Type 2	B	B	B-B	2 (0.12)	10 (0.53)	2 (0.18)	7 (1.00)		30 (0.83)	26 (0.66)	7 (0.44)	1 (0.04)			85 (0.39)
Type 3	AB	AB	AB-AB	1 (0.06)	3 (0.16)	1 (0.09)				1 (0.03)					6 (0.03)
Type 4	A	AB	AB-AB							2 (0.05)					2 (0.01)
Type 5	B	AB	AB-AB										1 (0.06)		1 (0.005)
Type 6	AB	A	AB-AB	3 (0.18)	5 (0.26)										8 (0.04)
Type 7	A	AB	A-A					2 (0.29)					4 (0.25)		6 (0.03)
Type 8	AB	A	B-B									1 (0.04)			1 (0.005)
Type 9	AB	B	B-B		1 (0.05)				1 (0.03)	3 (0.08)					5 (0.02)
Type 10	B	AB	B-B	1 (0.06)											1 (0.005)
Type 11	A	B	A-A					1 (0.14)							1 (0.005)
Type 12	A	B	B-B								2 (0.13)				2 (0.01)
Type 13	B	A	B-B	4 (0.23)											4 (0.02)
Type 14	B	A	A-A										1 (0.06)		1 (0.005)
Type 15	A	A	B-A									8 (0.32)			8 (0.03)
Type 16	B	B	B-A							4 (0.10)					4 (0.02)
Type 17	B	B	A-B								1 (0.06)				1 (0.005)
n[c]				17 (a)	19 (b)	11 (a)	7 (a)	7 (a)	36 (cd)	39 (ce)	16 (f)	25 (ag)	16 (ah)	24 (i)	217

[a] Defined by the RPA pattern for probes representing either the three gRNAs of subgroup II CMV or the four ORFs in the three gRNAs of subgroup I CMV.
[b] *X-Y* denotes the RPA pattern with probes I.3 and I.4, respectively.
[c] Number of isolates analyzed. The same letter (in parentheses) indicates that there was no significant differences in genetic composition between both populations in a χ^2 test.

transmission efficiencies reported for CMV (11, 42), the probability of more than one aphid in the group transmitting the virus (0.03) was smaller than the probability that one aphid transmitted the virus (0.1). Table 2 shows that these probabilities, calculated as described by Swallow (53), held for our experimental conditions, since 64 of 250 inoculated plants were infected. Virion RNA was purified from the 64 infected plants, and RPA was done with probes I.1, I.2, and I.4. Most (80%) of the infected plants showed a type 1 isolate, and no plant was found to be infected by type 2 (Table 2). The other plants were infected by reassortants of types 11 (1%) and 14 (1%), and, mainly, of a type (AAB; 16%) not found in the field. Also, a plant showed the partial mixed type 7 (1%). Thus, even if the type composition found for this experimental population differs from that in natural ones, both data sets agree in showing that association of gRNA1, -2, and -3 of RPA pattern A or B does not occur at random and in suggesting that reassortment between types 1 and 2 seems not to be favored.

In natural populations, no mixed infections or reassortants between CMV isolates in subgroups I and II were found (Table 1).

Genetic exchange by recombination. Since two probes, I.3 and I.4, were used for RPA of RNA3 of subgroup I isolates, recombinant RNA3 generated by the exchange of the 5′ or 3′ portion (MP or CP ORF, respectively [40]) of RNA3 belonging to RPA pattern A or B could be detected (Fig. 2, lanes 9 to 13). Such recombinant RNA3s were found for 7% of subgroup I isolates (types 15 to 17 in Table 1) from three populations, BAR93, BAR94, and MAD93. The recombinant RNA3s identified were of both possible types: A-B (for MP-CP) and B-A (for MP-CP), though the latter type was far more frequent. Recombinant RNA3s were found in combination with RNA1 and -2 of either type A or type B (Table 1). In addition to pattern type A or B, RPA with probes I.3 and I.4 allows a more detailed differentiation of MP and CP ORFs by analyzing the presence or absence of protected fragments. For each of the 13 isolates with a recombinant RNA3, the RPA haplotype for each of probes I.3 (MP ORF) and I.4 (CP ORF) was different (not shown).

Constraints to genetic variation in CMV populations. Some of us have recently reported the sequence of the region limited by nucleotides 1389 to 1840, in the CP ORF, of RNA3 in 12 field CMV isolates (2). These isolates were randomly chosen between type 1 and type 2 isolates of the populations collected before 1993, to compare six type 1 and six type 2 isolates. To obtain information on sequence variation of the other two gRNAs, we have determined the sequences of two regions, randomly chosen, one in RNA1 (nucleotides 1159 to 1435) and one in RNA2 (nucleotides 1370 to 1680). These new sequenced regions were determined for 10 isolates, 6 of type 1 and 4 of type 2. These sequences are available from EMBL/GenBank/DDBJ data bases under accession numbers Z77138 to Z77157 for RNA1 and -2 and X81161 to X81169 for RNA3.

Sequence data were used to make a clustering analysis by the neighbor-joining method of Saitou and Nei (50). For each of the three gRNAs, clusters corresponded to the RPA patterns A and B with a bootstrap confidence of $P \leq 0.001$ (not shown).

TABLE 2. Aphid transmission of CMV from tomato plants infected with isolates VAL.T89/21 (type 1) and VAL.T89/5 (type 2)[a]

Haplotype	RPA pattern			No. (frequency)
	RNA1	RNA2	RNA3	
1	A	A	A	51 (0.80)
7	A	AB	A	1 (0.01)
11	A	B	A	1 (0.01)
14	B	A	A	1 (0.01)
18	A	A	B	10 (0.16)

[a] Of 250 plants inoculated with groups of three apterae females of *M. persicae*, 64 were infected. The probability of transmission by a single aphid (53) was 0.094.

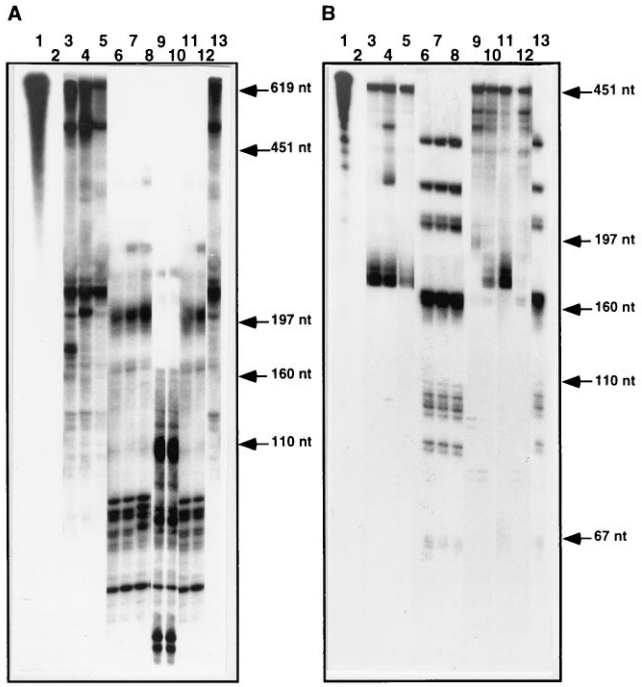

FIG. 2. RPA patterns for RNA3 of CMV field isolates from populations MAD93, BAR93, and BAR94. RPAs were done with probes I.3 (A) and I.4 (B). Lane 1, unhybridized probe; lane 2, unhybridized probe, RNase digested. Lanes 3 to 13 present patterns for the following isolates (with the indicated pattern for probes I.3 and I.4): lane 3, MAD.T93/2 (A-A); lane 4, MAD.Z93/8 (A-A); lane 5, MAD.Z93/9 (A-A); lane 6, BAR.T93/47 (B-B); lane 7, BAR.T93/6 (B-B); lane 8, BAR.T93/7 (B-B); lane 9, MAD.M93/9 (B-A); lane 10, MAD.Z93/1 (B-A); lane 11, BAR.T93/16 (B-A); lane 12, BAR.T93/19 (B-A); lane 13, BAR.T94/84 (A-B). The electrophoretic mobility of molecular size markers is indicated to the right of each panel. nt, nucleotides.

Levels of sequence diversity were 8.2% for RNA1, 9.6% for RNA2, and 6.9% for RNA3. These values were an order of magnitude smaller when sequences within type 1 or 2 were compared (not shown). Table 3 shows nucleotide diversity values calculated for nonsynonynous (d_{NS}) and for synonynous (d_S) sites when each sequence from one type was compared with each sequence from the other type ("all" in Table 3) and when only sequences within type 1 or 2 were compared ("within" in Table 3). The data in Table 3 are the average values for these comparisons. "All" values (among-type comparisons) represent the nucleotide changes that have occurred in the phylogenetic lines leading from the common ancestor of both types 1 and 2. "Within" values (within-type comparisons) represent the changes that have occurred during the divergence of each type from its immediate ancestor. When among-type comparisons were done, diversities at nonsynonymous and at synonymous sites were similar for RNA1 and -2 and somewhat smaller for RNA3. The d_{NS}/d_S ratio is an estimation of the degree of selective constraint to the evolution of the encoded protein. For all three gRNAs, these ratios were of the same order, though lower for RNA3. When d_{NS}, d_S, and d_{NS}/d_S were calculated for sequence comparisons within the same type 1 or 2, this was no longer so: for RNA2 and -3, the d_{NS}/d_S ratios were similar to those obtained before, but for RNA1, the ratio was more than five times higher. This result indicates that the evolutionary constraints imposed on the encoded sequences for RNA1 were different, and higher, during the period of divergence between types 1 and 2 than they have been since the splitting of both types. For RNA2 and -3, no such differences in the degree of selection were evidenced.

DISCUSSION

CMV is a multipartite plant virus that has very successfully colonized a large number of host plants in most regions of the world. The very numerous CMV strains described have been classified into two subgroups, I and II, based on serology and on molecular hybridization of the gRNA (40). The RPA characterization of the gRNA of 217 isolates representing 11 CMV field populations sampled in different years and at different locations in Spain shows that a large majority of isolates belong to subgroup I. Subgroup II isolates were found only in autumn samples, mostly from northern locations, in agreement with their lower thermal optima compared with subgroup I (30). This has also been reported from other countries (12, 43, 49). No mixed infections with isolates belonging to both subgroups were found, nor were isolates with reassortant genomes derived from subgroups I and II.

RPA showed that RNA1, -2, and -3 of subgroup I isolates belong to two clearly different genetic types, A and B, which was confirmed by sequence analysis. Differences among sequences between types were about 7 to 10%, while differences within types were an order of magnitude smaller. On the basis of these RPA patterns for each gRNA, haplotypes for the isolates were defined. The frequency compositions for these types differed significantly for the sampled populations, without correlation between genetic composition of the population and year, location, or host plant sampled. This random fluctuation of the genetic structure of CMV populations could be explained by population bottlenecks associated with seasonal crashes of the populations of host crop plants and/or aphid vectors, plus recolonizations from local or distant reservoirs. A similar population structure has been described for the CMV vectors *M. persicae* and *Aphis gossypii* (31). Thus, the genetics and dynamics of CMV populations correspond to a metapopulation structure, with local extinctions and random recolonizations, as has also been described in the few analyzed instances for other plant microparasites (54).

A significant proportion (16%) of the analyzed isolates in subgroup I represented mixed infections with isolates of the two prevalent genetic types, 1 and 2. Mixed infections are a condition for, and may lead to, genetic exchange between different genetic types, and genetic exchange both by reassortment and by RNA recombination was seen to occur. Isolates

TABLE 3. Nucleotide diversities for RNA1, RNA2, and RNA3 of CMV[a]

Comparison	Nucleotide diversity		
	RNA1	RNA2	RNA3
All			
d_{NS}	0.00606	0.00699	0.00430
d_S	0.30092	0.22185	0.08577
d_{NS}/d_S	0.020	0.032	0.050
Within			
d_{NS}	0.00629	0.00071	0.00091
d_S	0.00532	0.03239	0.01303
d_{NS}/d_S	0.118	0.022	0.070

[a] Nucleotide diversities, defined here as average number of nucleotide substitutions per site, were computed separately for nonsynonymous (d_{NS}) and synonymous (d_S) sites by the PBL method. Data are from all possible two-by-two comparisons (all) or only from comparisons within sequences of the same type A or B (within) presented as averages.

with reassortant genomes have been found in natural populations of multipartite plant RNA viruses (48, 56) as well as of segmented animal RNA viruses (20, 33). Indeed, the possibility of reassortment among the segments of a divided genome has been proposed as the evolutionary advantage that would compensate for the fitness loss intrinsic to a divided genome, either by generating new, fitter genetic combinations or by compensating for the deleterious effects of mutation accumulation (reviewed in reference 9). These hypotheses have been tested under experimental conditions, and mutation accumulation has been shown to lead, both for segmented and for nonsegmented RNA viruses, to a fitness loss when the viral population was subjected to repeated bottlenecks (7, 15, 37). For the segmented RNA phage ϕ6, reassortment could reverse this fitness loss (10). Nevertheless, theories on genetic exchange and the evolution of segmentation in RNA viruses need to be tested with data from natural populations. Henderson et al. (20) have recently reported data on the genetic structure of natural populations of Sin Nombre hantavirus in California and Nevada. Two main genetic lineages were found for each of the three genomic RNAs. Isolates with reassortant genomes represented a large proportion (~50%) of the populations. Reassortant isolates did not correspond to random reassociation of the three genomic RNAs, which has also been reported for bunyaviruses under experimental conditions (4, 55). The most frequent reassortants were found in most sampled populations, indicating a good competitive ability regarding the parent genotypes. The work presented here is, to our knowledge, the first quantitative report of reassortment in natural populations of a multipartite virus. As for the bunyaviruses, for CMV, reassortant genomes do not represent a random association of the three genomic RNAs, neither in natural field populations (Table 1) nor under experimental conditions (Table 2). This may indicate that for divided genomes, some genetic associations are intrinsically favored over others. In addition, in natural conditions there may be selective factors that operate on the possible reassortants. For CMV, one such factor, suggested by the data in Table 2 as well as from the literature (42), may be selection by the aphid vector. On the other hand, our data differ from those of Henderson et al. (20) in the important point that for CMV populations, reassortants were 4% of the population, did not become established, and, for each type, were not found in more than one population. Thus, it seems that selective factors favor the association of the three genomic RNAs of CMV of either type A or B in the haplotypes that we have called here 1 and 2. The nature of these factors is not known at present. An obvious possibility would invoke the optimization of protein-protein or protein-RNA interactions, and several such interactions could play an important role. For instance, proteins p1a and p2a, encoded by RNA1 and -2, must interact in the *Bromoviridae* (to which CMV belongs) for the replicase complex to be active (23), and the genomic RNAs must interact with the MP, encoded by RNA3, for movement of the viral infection from cell to cell (13) and with the CP for encapsidation and aphid transmission (11). The degree of divergence for p1a, p2a, and CP, shown by the d_{NS}/d_S ratios, is about 10 times smaller than those reported for a large range of DNA-encoded proteins (36), for the 126/183K ORF of tobacco mild green mosaic tobamovirus (17), and for nonantigenic sites of the hemagglutinin gene of influenza A orthomyxovirus (21) and is similar to those reported for the core protein gene of hepatitis C virus (22). Thus, d_{NS}/d_S ratios found for CMV coding regions are similar to those reported for proteins that are under high evolutionary constraint. The data in Table 3 indicate, moreover, that constraints to the divergence of CMV-encoded proteins may have changed during the evolutionary history of CMV types 1 and 2, as shown by the values for RNA1 when all or only within-type sequence comparisons were done. This finding is compatible with protein-protein and/or RNA-protein interactions having a role in CMV evolution, but the weight of each possible interaction may have varied during CMV's evolutionary history.

Recombinants were also found for RNA3, and the frequency of isolates with recombinant RNA3 is similar to that of reassortants. RNA recombination has frequently been reported in experimental and field populations of animal and plant viruses (for reviews, see references 25 and 52), but this is the first quantitation of the frequency of recombinants in natural populations of an RNA virus. We have also recently reported the frequency of recombinants for field populations of the noncoding satellite RNA of CMV (3). Although each recombinant RNA3 detected had a different RPA pattern for each of probes I.3 and I.4, we do not know if all recombinants in the same population derived from a single recombination event, followed by divergence by mutation accumulation, or were generated by different recombination events. What we want to stress here is that the frequency of genetic exchange by RNA recombination is no less in CMV than by segment reassortment. Thus, our data do not support the purifying selection model hypotheses on the evolution of RNA divided genomes, proposed to counteract the Müller's ratchet hypothesis (9). It could be argued that CMV populations are so large that Müller's ratchet would not operate and, thus genetic exchange would not be a biological advantage for this virus. Most probably this is not the case: CMV populations pass through severe bottlenecks during aphid transmission from plant to plant (5) as well as during the seasonal local collapses indicated by their genetic structure (Table 1). Nor do our present data support other hypotheses proposed for the evolution of divided RNA genomes (26, 34).

The data here presented may also be relevant to another aspect of virology. Transgenic plants resistant to virus diseases through pathogen-derived resistance (18) may be an efficient mean of disease control. A warning against their widespread use has been based on the grounds that recombination between the infecting virus and the transgene may have important effects on the evolution of viral populations. Genetic exchange may have profound evolutionary consequences, as has been shown for some viral systems (33). Our data, though, show that although mixed infections are frequent in CMV populations, and RNA reassortment and recombination may also be frequent, reassortant and recombinant isolates are infrequent, indicating that most heterologous genetic combinations seem to be at a competitive disadvantage.

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